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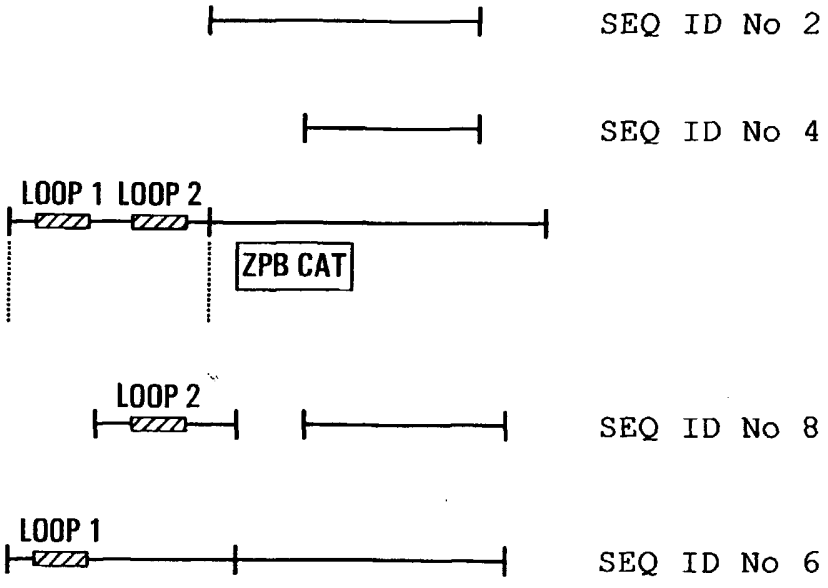

(57) Abstract: The present invention provides immunocontraceptive vaccines comprising a zona pellucida (ZP) polypeptide, and/or a variant thereof, from a carnivorous mammal such as cat, dog, ferret or mink. Such vaccines are useful in reducing fertility of cats and/or dogs.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

ANTIGENS FOR IMMUNOCONTRACEPTION

This application claims priority from US 60/354,525 filed February 8, 2002 and US 60/380,293 filed May 15, 2002, the contents of which are herein incorporated by reference.

Field of the Invention

The present invention relates to the field of immunology, in particular, to immunocontraceptive vaccines.

Background of the Invention

Increasing populations of feral or stray domestic dogs and cats has been a growing problem in North America and the rest of the world. For example, an estimated 40% of domestic cats (*Felis catus*) in the United States are classified as feral or stray.

Concerns about impacts on wildlife, transmission of infectious diseases, and the welfare of the cats and dogs themselves have led to various strategies to reduce the number of feral cats and dogs. Locally, and throughout the world, extermination has been the dominant method used in the attempt to control free-ranging feral cats and dogs.

Surgical sterilisation of feral cats and dogs by veterinarians followed by release back into the colony has been increasingly utilised as a humane tool to lower feral cat and dog populations in the last 2 decades. Despite the success of large-scale surgical sterilisation, such programs are not financially or logistically feasible in many locations.

During the last decade, interest has increased in applying immunocontraception (IC) as a reliable method to

lower population of pest species. IC can be a humane means of reducing fertility in domestic, feral and wild mammals (Oogjes, 1997), and several potential IC targets exist. For example, a vaccine that used gonadotrophin-releasing hormone (GnRH) as antigen, depressed ovarian activity in horses for one breeding season (Bradley *et al.*, 1999). The difficulty with GnRH directed vaccines is that there is a potential for endocrine dysfunction (Muller *et al.*, 1997). Zona pellucida (ZP), a noncellular glycoprotein coat surrounding the mammalian egg, regulates sperm-egg interaction during fertilisation (Sacco and Yurewicz, 1989). This structure is an ideal candidate for a contraceptive target, since altering its structure can prevent pregnancy. ZP immunisation has been effective in lowering fertilisation rates of many mammals (Willis *et al.*, 1994; Kirkpatrick *et al.*, 1996; 1996; Brown *et al.*, 1997a,b; Harris *et al.*, 2000). Two independent reports have indicated that pig zona pellucida (pZP) is an effective immunocontraceptive (although requires multiple boosters) in domestic cats (Ivanova *et al.*, 1995; Bradley *et al.*, 1999). Porcine zona pellucida has also been used in liposome-based immunocontraceptive vaccines for reducing fertility of certain mammals by 90-100% with a multi-year efficacy (Brown *et al.*, 2001). However, use of pZP in such a liposome-based vaccine as a single administration vaccine for cats is ineffective in cats.

Summary of the Invention

There is described an immunocontraceptive vaccine for cats and/or dogs comprising a zona pellucida polypeptide, and/or a variant thereof, from a carnivorous mammal and a physiologically acceptable auxiliary.

There is further described a method for reducing fertility in cats and/or dogs comprising administering to a cat or a dog an immunocontraceptive vaccine comprising a zona pellucida polypeptide, and/or a variant thereof, from a carnivorous mammal and a physiologically acceptable auxiliary.

There is still further described the use of a zona pellucida polypeptide, and/or a variant thereof, from a carnivorous mammal for reducing fertility in cats and/or dogs or for preparing a medicament for reducing fertility in cats and/or dogs.

There is yet further described a commercial package comprising a zona pellucida polypeptide, and/or a variant thereof, from a carnivorous mammal together with instructions for its use in reducing fertility in cats and/or dogs.

There is still yet further described an isolated DNA molecule that codes for a zona pellucida polypeptide, and/or a variant thereof, from a ferret or mink.

There is also described a zona pellucida polypeptide, or a variant thereof, from a ferret or mink.

There is also described an isolated polypeptide comprising a sequence selected from the group consisting of:

(a) SEQ ID NO: 8;

(b) SEQ ID NO: 6;

(c) SEQ ID NO: 4;

(d) SEQ ID NO: 2;

(e) an amino acid sequence which is substantially identical to any one of (a) to (d); and

(f) an immunologically active fragment of at least 12 amino acids in length of any one of (a) to (e).

There is also described an isolated DNA encoding the polypeptide described above.

There is also described a composition comprising the polypeptide described above and a carrier or diluent suitable for use in a vaccine.

There is also described an expression vector comprising the DNA described above.

There is also described a host or host cell comprising the expression vector described above.

There is also described a kit for inducing infertility in a mammal comprising the polypeptide described above and instructions for its use in eliciting an immune response against native zona pellucida in a mammal.

There is also described a method for inducing anti-ZPB antibodies in a mammal, the method comprising administering to the mammal at least one polypeptide described above, wherein said administering induces production of an antibody that binds mammalian zona pellucida.

There is also described a method for inducing infertility in a mammal comprising administering to the mammal at least one polypeptide described above.

There is also described a method of inducing infertility in a mammal comprising administering at least one polypeptide described above, wherein said administering

induces production of an antibody that binds mammalian zona pellucida.

There is also described a method of producing the polypeptide described above comprising culturing the host or host cell described above.

There is also described an antibody immunoreactive to the polypeptide described above.

There is also described the antibody described above which is immunoreactive against at least 2 native zona pellucida.

Brief Description of the Drawings

The invention will now be described by way of example having regard to the appended drawings in which:

Figure 1 is a graph showing the production of anti-SIZP antibodies by rabbits immunised with porcine zona pellucida (pZP) or cat zona pellucida (cZP) encapsulated in liposomes with either FCA or alum adjuvant as a single administration delivery system.

Figure 2 is a Western blot of a gel electrophoresis of dZP (lanes A and B), feZP (lanes C and D), cZP (lane E) and pZP (lanes F and G) probed with rabbit anti-cZP antibodies showing the cross-reactivity of rabbit anti-cZP antibodies to cZP, dZP, pZP, and feZP.

Figure 3 is a Western blot of a gel electrophoresis of mZP (lanes A, B, and C) probed with rabbit anti-pZP antibodies (lane A) and rabbit anti-cZP antibodies (lanes B and C) showing the cross-reactivity of rabbit anti-cZP and anti-pZP antibodies to mZP.

Figure 4 shows an alignment of a number of mammalian zona pellucida sequences.

Figure 5 shows alignments of specific zona pellucida sequences between various species.

Figure 6 is a schematic depiction of the alignment of the zona pellucida sequences.

Detailed Description of the Preferred Embodiments

Zona pellucida (ZP) polypeptides have now been identified that act as antigens to induce the production of antibodies with a high affinity for cat and dog zona pellucida and hence cat and dog oocytes. Since immunocontraception (IC) based on use of ZP antigens relies on a balance between antigenicity of foreign ZP and the ability of antibodies raised against the foreign ZP to bind to the ZP on the targeted oocyte surface, zona pellucida from animals more closely related to cats and/or dogs, than is the pig, could prove useful in IC of cats. It has now been found that ZP antigens from carnivorous mammals are particularly useful in preparing immunocontraceptive vaccine that are capable of producing immune responses in cats and/or dogs. ZPB is particularly useful as the antigen. In particular, the carnivorous mammals may be selected from the group consisting of cat (e.g. *Felis catus*), dog (e.g. *Canis familiaris*), ferret (e.g. *Mustela putorius furo*), and mink (e.g. *Mustela vison*). Thus, cat ZP (cZP), dog ZP (dZP), ferret ZP (feZP), mink ZP (mZP) and/or variants thereof are particularly useful as antigens in the immunocontraceptive vaccine. More particularly, cat ZPB (cZPB), dog ZPB (dZPB), ferret ZPB (feZPB), mink ZPB (mZPB) and/or variants thereof are preferred.

The term 'variants' means recombinant or denatured proteins or peptides, or fragments thereof, or fragments of native ZP, which are capable of producing the desired immune response in cats and/or dogs. Substitutions, additions and/or deletions of native or recombinant ZP are encompassed by variants. Variants are generally at least 50% homologous to native ZP. Variants having homology of at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90% or 95% to the native ZP are also particularly contemplated within the scope of the invention. Fragments of native, recombinant or denatured ZP proteins or peptides are generally at least 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 44, 46, 48 or 50 amino acids in length. Preferably, such fragments include amino acids

VSTTQSPGTSRPPTPASRVTPQ (amino acid numbers 29 to 50 of cat zona pellucida), and

PRNPPDQALVSSLSPS (amino acid numbers 79 to 94 of cat zona pellucida), and

VRTTQSPQMLRTPAPPSGVTPQ (from SEQ ID NO 6), and

PTLLSSLSYSPDQNR (from SEQ ID NO 8).

The polypeptides of the invention include any combination of the above fragments and their consensus sequences.

The term "isolated polynucleotide" is defined as a polynucleotide removed from the environment in which it naturally occurs. For example, a naturally-occurring DNA molecule present in the genome of a living bacteria or as part of a gene bank is not isolated, but the same molecule separated from the remaining part of the bacterial genome, as a result of, *e.g.*, a cloning event (amplification), is isolated. Typically, an isolated DNA molecule is free from

DNA regions (*e.g.*, coding regions) with which it is immediately contiguous at the 5' or 3' end, in the naturally occurring genome. Such isolated polynucleotides may be part of a vector or a composition and still be defined as isolated in that such a vector or composition is not part of the natural environment of such polynucleotide.

The present invention includes amino acid sequences which are homologous to SEQ ID NOS: 2, 4, 6 and 8, and the fragments above. As used herein, "homologous amino acid sequence" is any polypeptide which is encoded, in whole or in part, by a nucleic acid sequence which hybridizes at 25-35°C below critical melting temperature (Tm), to any portion of the nucleic acid sequence of SEQ ID NOS: 1, 3, 5 or 7. A homologous amino acid sequence may be one that differs from an amino acid sequence shown in SEQ ID NOS: 2, 4, 6 or 8 by one or more conservative amino acid substitutions.

Homologous amino acid sequences include sequences that are identical or substantially identical to SEQ ID NOS: 2, 4, 6 or 8. By "amino acid sequence substantially identical" is meant a sequence that is at least 90%, preferably 95%, more preferably 97%, and most preferably 99% identical to an amino acid sequence of reference and that preferably differs from the sequence of reference by a majority of conservative amino acid substitutions.

Conservative amino acid substitutions are substitutions among amino acids of the same class. These classes include, for example, amino acids having uncharged polar side chains, such as asparagine, glutamine, serine, threonine, and tyrosine; amino acids having basic side chains, such as lysine, arginine, and histidine; amino acids having acidic side chains, such as aspartic acid and

glutamic acid; and amino acids having nonpolar side chains, such as glycine, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan, and cysteine.

Homology is measured using sequence analysis software such as Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705. Amino acid sequences are aligned to maximize identity. Gaps may be artificially introduced into the sequence to attain proper alignment. Once the optimal alignment has been set up, the degree of homology is established by recording all of the positions in which the amino acids of both sequences are identical, relative to the total number of positions.

Homologous polynucleotide sequences are defined in a similar way. Preferably, a homologous sequence is one that is at least 45%, more preferably 50%, 55%, 60%, 65%, 70%, 75%, 80%, and even more preferably 85%, 87%, 90%, 93%, 96% and most preferably 99% identical to SEQ ID NOS: 1, 3, 5 or 7.

As used herein, a fusion polypeptide is one that contains a polypeptide or a polypeptide derivative of the invention fused at the N- or C-terminal end to any other polypeptide. A simple way to obtain such a fusion polypeptide is by translation of an in-frame fusion of the polynucleotide sequences, *i.e.*, a hybrid gene. The hybrid gene encoding the fusion polypeptide is inserted into an expression vector which is used to transform or transfect a host cell. Alternatively, the polynucleotide sequence encoding the polypeptide or polypeptide derivative is inserted into an expression vector in which the

polynucleotide encoding the peptide tail is already present. These and other expression systems provide convenient means for further purification of polypeptides and derivatives of the invention. Alternatively, various fragments of the polypeptides of the invention may be fused together to produce chimeric polypeptides.

Accordingly, a second aspect of the invention encompasses (i) an expression cassette containing a DNA molecule of the invention placed under the control of the elements required for expression, in particular under the control of an appropriate promoter; (ii) an expression vector containing an expression cassette of the invention; (iii) a procaryotic or eucaryotic cell transformed or transfected with an expression cassette and/or vector of the invention, as well as (iv) a process for producing a polypeptide or polypeptide derivative encoded by a polynucleotide of the invention, which involves culturing a procaryotic or eucaryotic cell transformed or transfected with an expression cassette and/or vector of the invention, under conditions that allow expression of the DNA molecule of the invention and, recovering the encoded polypeptide or polypeptide derivative from the cell culture.

A recombinant expression system is selected from procaryotic and eucaryotic hosts. Eucaryotic hosts include yeast cells (*e.g.*, *Saccharomyces cerevisiae* or *Pichia pastoris*), mammalian cells (*e.g.*, COS1, NIH3T3, or JEG3 cells), arthropods cells (*e.g.*, *Spodoptera frugiperda* (SF9) cells), and plant cells. A preferred expression system is a procaryotic host such as *E. coli*. Bacterial and eucaryotic cells are available from a number of different sources including commercial sources to those skilled in the art, *e.g.*, the American Type Culture Collection (ATCC; Rockville, Maryland). Commercial sources of cells used for recombinant

protein expression also provide instructions for usage of the cells.

Antigens of the present invention may be formulated into vaccines in a number of ways. Methods of formulating vaccines in general are well known to those skilled in the art (for example, see Harlow *et al.*, 1988 the disclosure of which is herein incorporated by reference). Ivanova *et al.*, 1995; Bradley *et al.*, 1999; and Brown *et al.*, 2001, the disclosures of which are herein incorporated by reference, specifically disclose methods of formulating ZP antigens into a vaccine. Immunocontraceptive vaccines comprising the ZP antigens of the present invention may be formulated as either single or multiple administration vaccines. Single administration vaccines using a system such as that described in Brown *et al.*, 2001 are preferred.

The amount of ZP antigen used in a dose of the immunocontraceptive vaccine can vary depending on the source of the antigen and the size of the cat or dog. One skilled in the art will be able to determine, without undue experimentation, the effective amount of antigen to use in a particular application. The amount typically used falls in the range from about 15 µg to about 2 mg per dose. Preferably, the range is from about 20 µg to about 2 mg per dose, more preferably from about 20 µg to about 200 µg, and even more preferably from about 40 µg to about 120 µg. Typically, the amount for a small animal is about 50 µg per dose while for a large animal it is about 100 µg per dose.

Physiologically acceptable auxiliaries for immunocontraceptive vaccines are generally known in the art. Auxiliaries include carriers, diluents, adjuvants and any other typical vaccine ingredients.

Carriers and/or diluents are generally well known in the art. Typically, aqueous solutions, aqueous emulsions of an oil such as mineral oil, and non-aqueous media such as pure mineral oil may be used as carriers and/or diluents.

Suitable adjuvants include alum, other compounds of aluminum, Bacillus of Calmette and Guerin (BCG), TiterMax™, Ribi™, Freund's Complete Adjuvant (FCA) and a new adjuvant disclosed by the United States Department of Agriculture's (USDA) National Wildlife Research Center on their web site at http://www.aphis.usda.gov/ws/nwrc/pzp.htm based on Johne's antigen. Alum, other compounds of aluminum, TiterMax™ and the new USDA adjuvant are preferred.

Alum is particularly preferred as the adjuvant. Alum is generally considered to be any salt of aluminum, in particular, the salts of inorganic acids. Hydroxide and phosphate salts are particularly useful as adjuvants. A suitable alum adjuvant is sold under the trade name, ImjectAlum™ (Pierce Chemical Company) that consists of an aqueous solution of aluminum hydroxide (45 mg/ml) and magnesium hydroxide (40 mg/ml) plus inactive stabilizers. Alum is a particularly advantageous adjuvant since it already has regulatory approval and it is widely accepted in the art.

The amount of adjuvant used depends on the amount of antigen and on the type of adjuvant. One skilled in the art can readily determine the amount of adjuvant needed in a particular application. For immunocontraception, a suitable quantity of ImjectAlum™ may range from 0.1 ml/dose of vaccine to 0.5 ml/dose.

Liposomes are another typical vaccine ingredient. The vaccines of the present invention may be formulated with

or without liposomes. However, use of liposomes offers certain advantages. Liposomes are completely closed lipid bilayer membranes containing an entrapped aqueous volume. Liposomes may be unilamellar vesicles (possessing a single bilayer membrane) or multilamellar vesicles (onion-like structures characterized by multimembrane bilayers, each separated from the next by an aqueous layer. Although any liposomes may be used, including liposomes made from archaebacterial lipids, particularly useful liposomes use phospholipids and unesterified cholesterol in the liposome formulation. The cholesterol is used to stabilize the liposomes and any other compound that stabilizes liposomes may replace the cholesterol. Other liposome stabilizing compounds are known to those skilled in the art. Phospholipids that are preferably used in the preparation of liposomes are those with at least one head group selected from the group consisting of phosphoglycerol, phosphoethanolamine, phosphoserine, phosphocholine and phosphoinositol.

The amount of lipid used to form liposomes depends on the antigen being used but is typically in a range from about 0.05 gram to about 0.5 gram per dose of vaccine. Preferably, the amount is about 0.1 gram per dose. When unesterified cholesterol is also used in liposome formulation, the cholesterol is used in an amount equivalent to about 10% of the amount of lipid. The preferred amount of cholesterol is about 0.01 gram per dose of vaccine. If a compound other than cholesterol is used to stabilize the liposomes, one skilled in the art can readily determine the amount needed in the formulation.

In one embodiment, the vaccine composition may be formulated by: encapsulating the antigen or an antigen/adjuvant complex in liposomes to form liposome-

encapsulated antigen and mixing the liposome-encapsulated antigen with a carrier. If an antigen/adjuvant complex is not used in the first step, a suitable adjuvant may be added to the liposome-encapsulated antigen, to the mixture of liposome-encapsulated antigen and carrier, or to the carrier before the carrier is mixed with the liposome-encapsulated antigen. The order of the process may depend on the type of adjuvant used. Typically, when an adjuvant like alum is used, the adjuvant and the antigen are mixed first to form an antigen/adjuvant complex followed by encapsulation of the antigen/adjuvant complex with liposomes. The resulting liposome-encapsulated antigen is then mixed with the carrier. (It should be noted that the term "liposome-encapsulated antigen" may refer to encapsulation of the antigen alone or to the encapsulation of the antigen/adjuvant complex depending on the context.) When another is used, the antigen may be first encapsulated in liposomes and the resulting liposome-encapsulated antigen is then mixed into the adjuvant in a carrier.

Liposome-encapsulated antigen may be freeze-dried before being mixed with the carrier. In some instances, an antigen/adjuvant complex may be encapsulated by liposomes followed by freeze-drying. In other instances, the antigen may be encapsulated by liposomes followed by the addition of adjuvant then freeze-drying to form a freeze-dried liposome-encapsulated antigen with external adjuvant. In yet another instance, the antigen may be encapsulated by liposomes followed by freeze-drying before the addition of adjuvant.

Formulation of the liposome-encapsulated antigen into a hydrophobic substance may also involve the use of an emulsifier to promote more even distribution of the liposomes in the carrier. Typical emulsifiers are well known in the art and include mannide oleate (Arlacel™ A),

lecithin, Tween™ 80, Spans™ 20, 80, 83 and 85. Mannide oleate is a preferred emulsifier. The emulsifier is used in an amount effective to promote even distribution of the liposomes. Typically, the volume ratio (v/v) of carrier to emulsifier is in the range of about 5:1 to about 15:1 with a ratio of about 10:1 being preferred.

Administration of the vaccine composition can be done by any convenient method. Vaccine compositions may be administered parenterally (including intramuscularly, sub-cutaneously) or rectally. Parenteral administration is preferred.

For parenteral application, particularly convenient unit dosage forms are ampoules. Techniques that deliver the vaccine by injection and by remote delivery using darts, spring loaded syringes with jab sticks, air/carbon dioxide powered rifles, Wester gun and/or Ballistivet™ biobullets and retain the biological activity are particularly preferred.

Examples

Materials and methods:

Ovaries from dogs and cats were obtained from veterinarians following spaying of pet cats and dogs. Pig, ferret, and mink ovaries were obtained from a commercial source. Soluble isolated ZP was prepared from these ovaries as described by Brown *et al.* (1997b), the disclosure of which is herein incorporated by reference, to yield cZP, dZP, feZP, mZP, and pZP. Vaccines were constructed from the designated soluble isolated ZP (SIZP). The SIZP was encapsulated in liposomes formed using soybean L-α-lecithin (Calbiochem-Novabiochem, San Diego, CA, USA) and cholesterol (Calbiochem-Novabiochem) in a ratio of 10:1. Single

administration vaccines were formulated with 1 of 2 adjuvants, i.e. with Freund's complete adjuvant (FCA) or with alum. Rabbits were immunised with a single dose of the vaccine with FCA containing SIZP (100 µg pZP or 50 µg cZP) encapsulated in multilamellar liposomes (0.1 g lecithin and 0.01 g cholesterol) that were suspended in saline (0.25 mL) and emulsified in FCA (0.25 mL). Less cZP than pZP was used in the vaccine formulation to conserve the limited quantity of cZP available. A single dose of the vaccine with alum contained pZP (100 µg) and alum (Imject®alum, Pierce Chemical Co., Rockford, IL, USA) encapsulated in multilamellar liposomes (0.1 g lecithin and 0.01 g cholesterol) that were suspended in saline (0.15 mL) and emulsified in mineral oil/mannide oleate (8.5:1.5, v:v, 0.25mL). Rabbits were immunised with pZP in either vaccine with FCA or vaccine with alum or with cZP in vaccine with FCA. Serum samples were taken monthly to measure the production of anti-SIZP antibodies. Production of anti-SIZP antibodies was measured as described by Brown *et al.* (1997b), the disclosure of which is herein incorporated by reference, using protein A/alkaline phosphatase.

Gel electrophoresis and Western blotting was used to measure the affinity of anti-cZP antibodies for cZP, feZP, dZP, mZP and pZP and anti-pZP antibodies for mZP as follows. Protein samples were loaded on SDS-PAGE (12%) and analysed by Western blotting. For Western blotting, electrophoresed proteins were transferred to PVDF (Amersham) paper and blocked in QuickBlocker™ (Chemicon). The transferred blots were probed with primary antibody at 1:500 to 1:1000 dilution of TBS-Tween™ overnight at 4°C. The blots were washed 5X with TBS-Tween™ and then incubated with peroxidase labelled secondary antibody (goat anti-rabbit Ig, Jackson, 1:8000 in TBS-Tween™) for 30 minutes at room

temperature. Blots were then washed 5X with TBS-Tween™ and signals were detected by chemiluminescence (Santa Cruz) using X-ray film.

The affinity of rabbit anti-cZP, fallow deer anti-pZP and cat anti-pZP for porcine or cat zona pellucida glycoproteins was measured by ELISA. Protein G/alkaline phosphatase (Calbiochem-Novabiochem, San Diego, CA, USA) was used to measure fallow deer antibodies whereas protein A/alkaline phosphatase (Sigma Chemical Co.) was used to measure cat and rabbit antibodies as follows. Briefly, 1 µg of either pZP or cZP in sodium carbonate/bicarbonate buffer (100 µL, 0.035 M, pH 9.6) was pipetted into each well of a 96-well ELISA plate and allowed to incubate for 1 hour at 37°C. Unbound pZP or cZP was removed, and the wells coated with gelatin (3% gelatin in TBST buffer - Tris,0.01 M; NaCl,0.15 M; 0.05 % Tween™ 20, pH 8.0) for 15 minutes at room temperature. The wells were then washed 5 times with TBST buffer to remove unbound gelatin. Serum samples (100 µL) were added in 2-fold dilutions using TBST from 1:50 to 1:6400 and incubated at 37°C for 1 hour. Unbound antibody and other serum proteins were removed by washing with TBST 5 times. Bound antibody was measured with protein A or protein G/alkaline phosphatase using a Dynatech™ ELISA plate reader at 405 nm. One row in each plate did not receive serum (antibody) and served as a blank. Another row in each plate received doubling dilutions of a reference rabbit anti-pZP serum. Titers were determined using the linear portion of the titration curve and all titers are expressed as a percentage of the reference serum to control for interassay variability.

Results:

Rabbits immunised with pZP or cZP produced similar anti-SIZP titers 2 months post-immunisation although the anti-cZP titer was lower than the anti-pZP titers obtained with either vaccine with FCA or vaccine with alum 1 month post-immunisation (Figure 1). This may be due to less antigen being placed in the vaccine (1/2 the content of pZP). One skilled in the art can predict that production of anti-cZP antibodies will continue to increase and yield a titer similar to the anti-pZP titers. Such titers have been shown to be immunocontraceptive in a variety of mammals. Therefore, it is expected that immunisation of cats using cZP or SIZP from animals closely related to cats such as other carnivores like ferret, mink, or dog will produce anti-SIZP antibodies in sufficient quantity to effect immunocontraception.

Measurement of cross-reactivity of rabbit anti-cZP antibodies indicates that these antibodies cross-react strongly with dZP, feZP but there is very little cross-reactivity with pZP (Figure 2). Zona pellucida glycoproteins (ZP glycoproteins) form bands between 63 and 83 kDa during electrophoresis. Dog, ferret, and cat ZP glycoproteins were strongly recognised by rabbit anti-cZP antibodies (see lanes A, B, C, D and E) but rabbit anti-cZP antibodies failed to recognise pZP. This result demonstrates that cZP contains more epitopes in common with dZP and feZP than with pZP and therefore there is more likelihood that antibodies raised against either dZP or feZP will cross-react with cZP and consequently bind more strongly with cat oocytes and thereby cause immunocontraception.

Measurement of cross-reactivity of rabbit anti-cZP antibodies indicates that these antibodies bind more strongly with mZP than with pZP (Figure 3). This suggests

that mZP shares more epitopes with cZP than pZP and therefore one skilled in the art can predict that mZP is a good candidate antigen for the immunocontraception of cats.

Cross-reactivity can also be measured by ELISA. When titers of two cat anti-pZP sera were measured using pZP, the titers were 41% and 15% of the reference serum. However, when titers of the same antisera were measured using cZP, the titers were 2% and 2% of the reference serum. This indicates that antibodies raised in cats against pZP have little affinity for cZP and consequently cat oocytes. When the titers of two rabbit anti-cZP sera were measured using pZP, the titers were 7% and 40% of the reference serum. However, when titers of the same antisera were measured using cZP, the titers were 32% and 200% of the reference serum. This indicates that only about 20% of antibodies raised against cZP have epitopes in common with pZP. Similarly, when titers of two fallow deer anti-pZP sera were measured using pZP the titers were 56% and 125% of the reference serum. However, when titers of the same antisera were measured using cZP, the titers were 2% and 2% of the reference serum indicating that few epitopes recognised by fallow deer immunised with pZP are found in cZP. One can conclude that the epitopes in pZP recognised by cats, rabbits and fallow deer are very different than the epitopes recognised in cZP. This suggests that only antigens that have epitopes in common with cZP will be effective in an immunocontraceptive vaccine for cats. Based on ELISA measurements of cross-reactivity, one skilled in the art would predict that feZP, dZP, mZP or cZP would be effective antigens in an immunocontraceptive vaccine for cats.

Determination of the partial DNA sequence of feZPB allows comparison with other DNA sequences. SEQ ID NO. 1 is

the partial ferret DNA sequence that codes for the equivalent of cat ZPB amino acid region 309-428. (Cat ZPB, including the leader sequence, is a total of 570 amino acids in length.)

(SEQ ID NO. 1):

gggtccgtca ctcgggacag tattttcagg cttcaagtta gctgcagcta cttgatcagc agcaatgcct cccaggttaa tgtccagatt tttacgctcc caccacccct tcctgaaacc caggctggac cccttactct ggaactcaag attgccaaag ataagcacta tgaatcctat tacactgcca gtgactaccc agtggtgaag ctgcttcggg atcccattta cgtggaggtg tctatccgcc acagaacaga cccctacctg gggctgttcc tccagcactg ttgggccaca cccagcctaa acccccaaca tcagcgccag tggcccatgc tggtcaatgg ctgcccttа

This ferret sequence was cloned by reverse transcription/degenerate PCR method. Primers were based on multiple alignments that included ZPB sequences from cat, cow, human, possum, mouse, rat and pig ZPB. A search of GenBank™ indicates that SEQ ID NO. 1 matches best with cZPB, suggesting that feZP will have many epitopes in common with cZP and therefore will be effective as an antigen in a cat immunocontraceptive vaccine.

The ferret partial amino acid sequence corresponding to the nucleotide sequence above is given by SEQ ID No. 2:

(SEQ ID NO. 2):

GSVTRDSIFR LQVSCSYLIS SNASQVNVQI FTLPPPLPET QAGPLTLELK IAKDKHYESY YTASDYPVVK LLRDPIYVEV SIRHRTDPYL GLFLQHCWAT PSLNPQHQRQ WPMLVNGCP

SEQ ID NO. 3 is the partial nucleotide sequence of Canine ZPB.

(SEQ ID NO. 3):

ggttccgtta cccgtgacag tattttcagg ctccgagtta gctgcagcta
ctctataagt agcaatgcct tcccagttaa tgtccacgtg tttacatttc
caccaccgca ttctgagacc cagcctggac ccctcactct ggaactcaag
attgccaagg ataagcacta tggttcctac tacactgctg gtgactaccc
agtggtgaag ctacttcggg atcccattta tgtggaggtc tctatccgcc
acagaacaga cccccacctg gggctgctcc tccattactg ttgggccaca
cccagcagaa acccacagca tcagccccag tggctcatgc tggtgaaagg
ctgccccta

The dog partial amino acid sequence corresponding to the nucleotide sequence above is given by SEQ ID NO. 4.

(SEQ ID NO. 4):

GSVTRDSIFR LRVSCSYSIS SNAFPVNVHV FTFPPPHSET QPGPLTLELK
IAKDKHYGSY YTAGDYPVVK LLRDPIYVEV SIRHRTDPHL GLLLHYCWAT
PSRNPQHQPQ WLMLVKGCP

SEQ ID NO. 5 is another partial ferret DNA sequence that codes for SEQ ID NO 6.

(SEQ ID NO 5):

GGCTGCGGTACCTGGGTAAGGGAAGGCCCAGGCAGCTCCATGGTGCTAGAAGCCTCTTAC
AGCGGCTGCTATGTCACCGAGTGGGTAAGGACCACCCAATCGCCACAAATGCTGCGAACC
CCTGCACCACCATCAGGGGTGACTCCCCAGGATCCCCACTATATCATGCTACTTGGAGTT
GAAGGAGCAGATGTGACTGGACGCAGCACGGTTACAAAGACAAAGCTGCTTAAGTGTCCT
GTGGATCCCCCAGCCCTAGATGCTCCAAACGCTGACCTGTGTGATTCTGTCCCAGTGTGG
GACAGGCTGCCATGTGCTCCTTCATCTATCAGTCAAAGAGATTGTGAGAAGGTTGGTTGC
TGCTACAATTTGGAGGCTAATTCCTGTTACTATGGAAACACAGTGACGTCCCACTGTACC
CAAGATGGCCACTTCTCCATTGTCGTGTCTCGGAAGGTGACCTCACCCCCACTGCTCTTA
AATTCTGTGCGCTTGGCCTTCAGGAATGACCATGAATGCACCCCTGTGATGACAACACAC
ACCTTTGCCACCTTTTGGTTTCCATTAAATTCCTGTGGTACCACAAGACGGATCATTGGA
GACTGGGTAGTATATGAAAATGAGCTGGTCGCAACTAGAGATGTGAGAGCTTGGAGCCAT
GGTTCTATCACCCGTGACAGTATTTTCAGGCTTCAAGTTAGCTGCAGCTACTTGATCAGC
AGCAATGCCTCCCAGGTTAATGTCCAGATTTTTACGCTCCCACCACCCCTTCCTGAAACC
CAGGCTGGACCCCTTACTCTGGAACTCAAGATTGCCAAAGATAAGCACTATGAATCCTAT
TACACTGCCAGTGACTACCCAGTGGTGAAGCTGCTTCGGGATCCCATTTACGTGGAGGTG
TCTATCCGCCACAGAACAGACCCCTACCTGGGGCTGTTCCTCCAGCACTGTTGGGCCACA
CCCAGCCTAAACCCCCAACATCAGCGCCAGTGGCCCATGCTGGTCAATGGCTGCCCTTA

(SEQ ID NO 6):

GCGTWVREGPGSSMVLEASYSGCYVTEWVRTTQSPQMLRTPAPPSGVTPQDPHYIMLLGV
EGADVTGRSTVTKTKLLKCPVDPPALDAPNADLCDSVPVWDRLPCAPSSISQRDCEKVGC
CYNLEANSCYYGNTVTSHCTQDGHFSIVVSRKVTSPPLLLNSVRLAFRNDHECTPVMTTH
TFATFWFPLNSCGTTRRIIGDWVVYENELVATRDVRAWSHGSITRDSIFRLQVSCSYLIS
SNASQVNVQIFTLPPPLPETQAGPLTLELKIAKDKHYESYYTASDYPVVKLLRDPIYVEV
SIRHRTDPYLGLFLQHCWATPSLNPQHQRQWPMLVNGCP

SEQ ID NO. 7 is another partial dog DNA sequence that codes for SEQ ID NO 8.

(SEQ ID NO 7):

TGCTCAGGTGTCCTAGGAATCCCCCAGACCCAACTTTGTTATCTAGCTTGAGTTACTCTC
CTGATCAAAACAGAGCCCTAGATGTTCCAAATGCTGATCTGTGTGACTTTGTCCCAGTGT
GGGACAGGCTGCCATGTGTTCCTTCACCCATCACTGAAGAAGACTGCAAGAAGATTGGTT
GCTGCTACAATTTGGAGGTGAATTTCTGTTATTATGGAAACACAGTGACCTCCCACTGTA
CCCAAGATGGCCACTTCT
gap
GGTTCCGTTACCCGTGACAGTATTTTCAGGCTCCGAGTTAGCTGCAGCTACTCTATAAGT
AGCAATGCCTTCCCAGTTAATGTCCACGTGTTTACATTTCCACCACCGCATTCTGAGACC
CAGCCTGGACCCCTCACTCTGGAACTCAAGATTGCCAAGGATAAGCACTATGGTTCCTAC
TACACTGCTGGTGACTACCCAGTGGTGAAGCTACTTCGGGATCCCATTTATGTGGAGGTC
TCTATCCGCCACAGAACAGACCCCCACCTGGGGCTGCTCCTCCATTACTGTTGGGCCACA
CCCAGCAGAAACCCACAGCATCAGCCCCAGTGGCTCATGCTGGTGAAAGGCTGCCCCTA

(SEQ ID NO 8):

LRCPRNPPDPTLLSSLSYSPDQNRALDVPNADLCDFVPVWDRLPCVPSPITEEDCKKIGC
CYNLEVNFCYYGNTVTSHCTQDGHF
gap
GSVTRDSIFRLRVSCSYSISSNAFPVNVHVFTFPPPHSETQPGPLTLELKIAKDKHYGSY
YTAGDYPVVKLLRDPIYVEVSIRHRTDPHLGLLLHYCWATPSRNPQHQPQWLMLVKGCP

It is apparent to one skilled in the art that many variations on the present invention can be made without departing from the scope or spirit of the invention claimed herein.

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CLAIMS:

1. An isolated polypeptide comprising a sequence selected from the group consisting of:

(a) SEQ ID NO: 8;

(b) SEQ ID NO: 6;

(c) SEQ ID NO: 4;

(d) SEQ ID NO: 2;

(e) an amino acid sequence which is substantially identical to any one of (a) to (d); and

(f) an immunologically active fragment of at least 12 amino acids in length of any one of (a) to (e).

2. An isolated DNA encoding the polypeptide according to claim 1.

3. A composition comprising the polypeptide according to claim 1 and a carrier or diluent suitable for use in a vaccine.

4. An expression vector comprising the DNA according to claim 2.

5. A host or host cell comprising the expression vector according to claim 4.

6. A kit for inducing infertility in a mammal comprising the polypeptide according to claim 1 and instructions for its use in eliciting an immune response against native zona pellucida in a mammal.

7. A method for inducing anti-ZPB antibodies in a mammal, the method comprising administering to the mammal at least one polypeptide according to claim 1, wherein said administering induces production of an antibody that binds mammalian zona pellucida.

8. A method for inducing infertility in a mammal comprising administering to the mammal at least one polypeptide according to claim 1.

9. A method of inducing infertility in a mammal comprising administering at least one polypeptide according to claim 1, wherein said administering induces production of an antibody that binds mammalian zona pellucida.

10. A method of producing the polypeptide according to claim 1 comprising culturing the host or host cell according to claim 5.

11. The method of any one of claims 7 to 9 wherein the mammal is cat.

12. The method of any one of claims 7 to 9 wherein the mammal is dog.

13. An antibody immunoreactive to the polypeptide according to claim 1.

14. The antibody of claim 13 which is immunoreactive against at least 2 native zona pellucida.

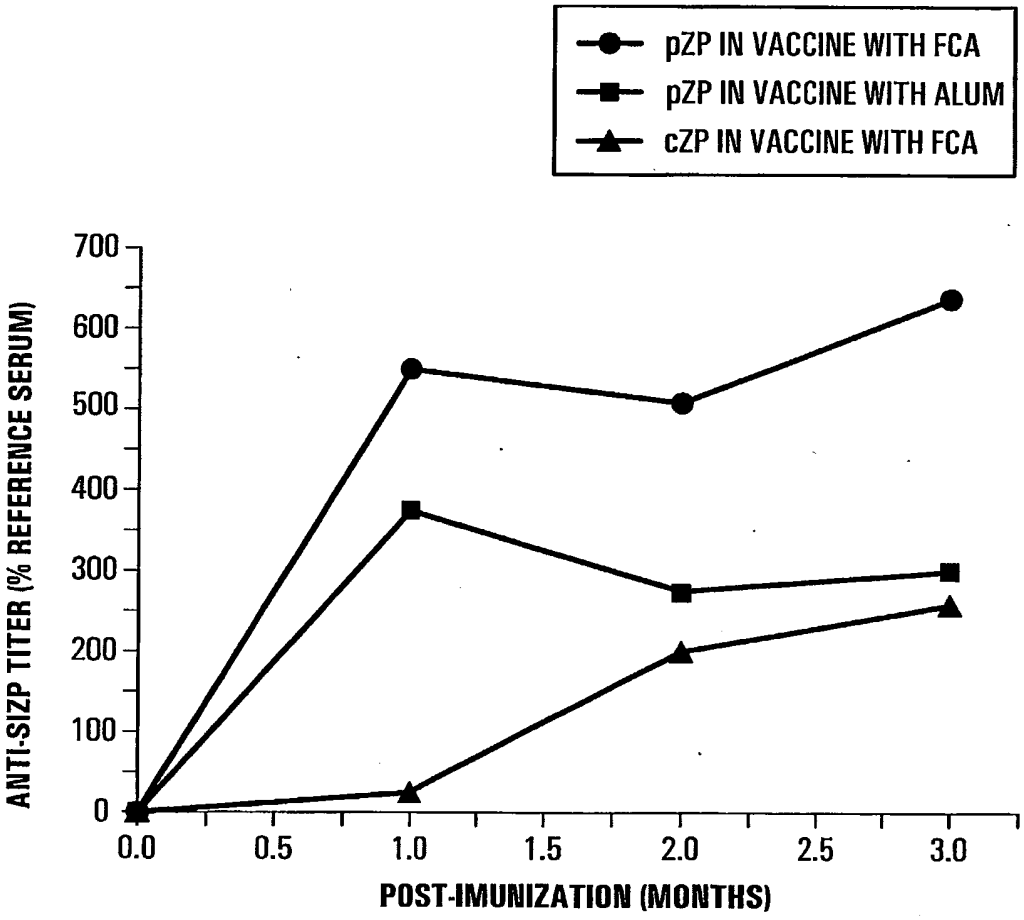

pZP IN VACCINE WITH FCA
pZP IN VACCINE WITH ALUM
cZP IN VACCINE WITH FCA
ANTI-SIZP TITER (% REFERENCE SERUM)
700
600
500
400
300
200
100
0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
POST-IMUNIZATION (MONTHS)

FIG. 1

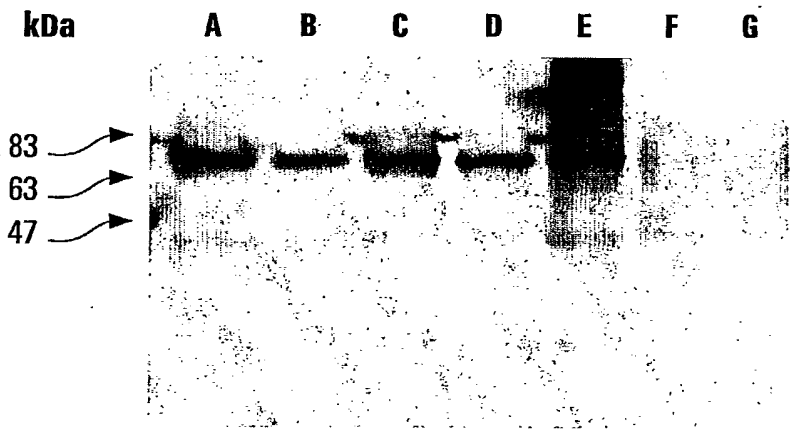


FIG. 2

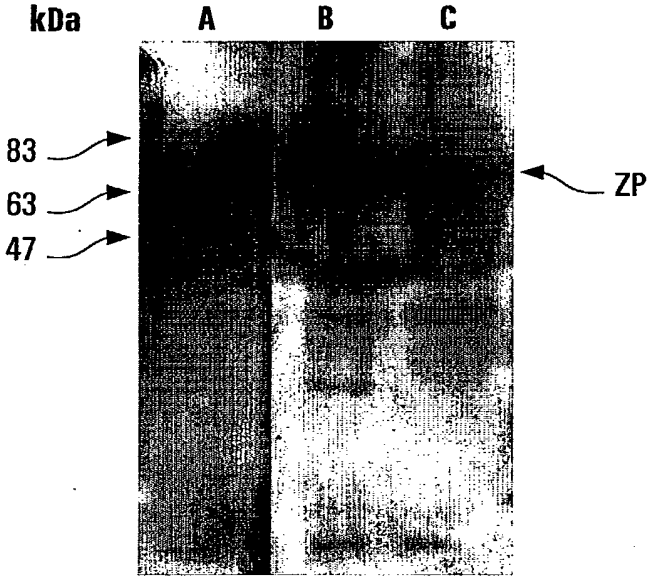


FIG. 3

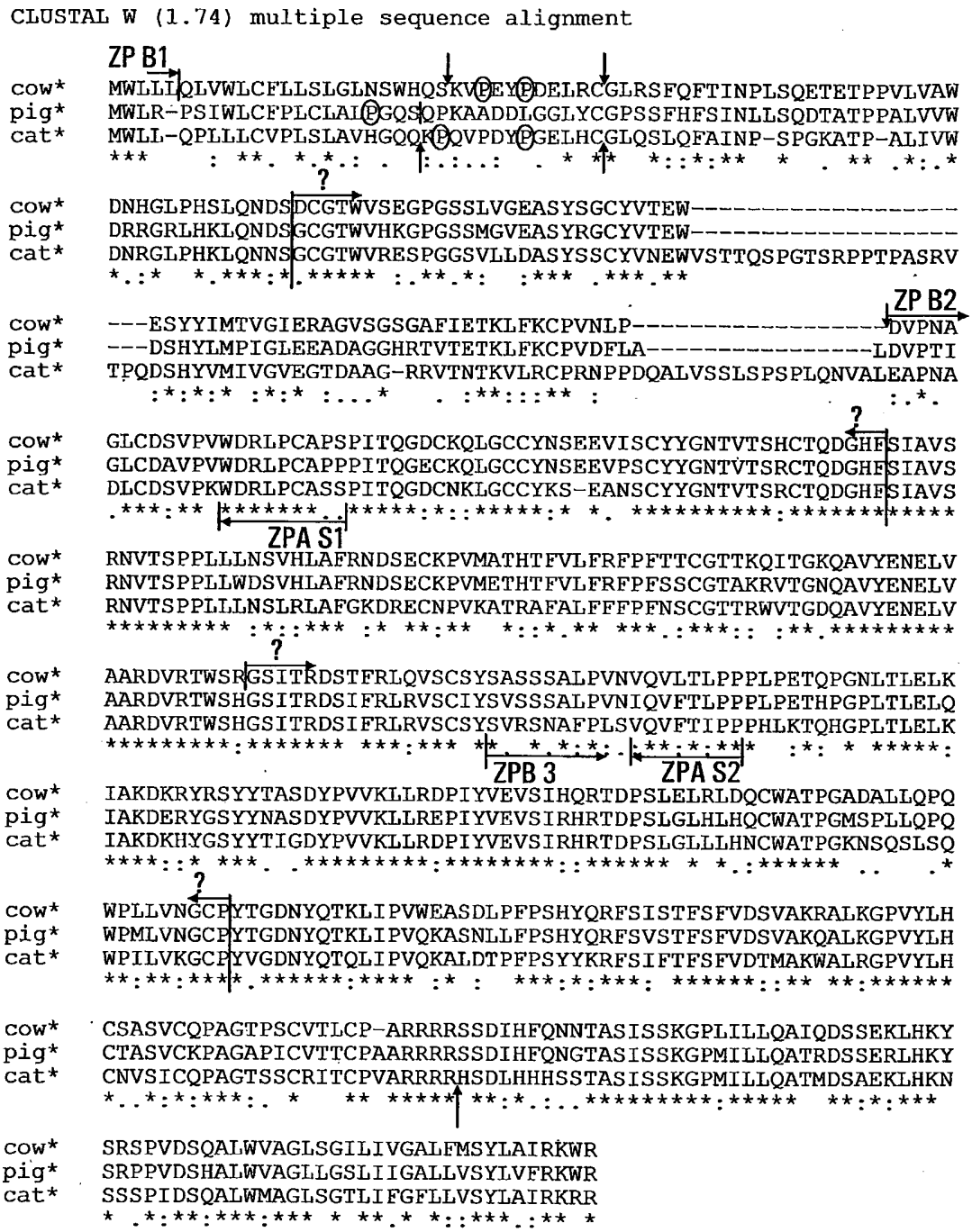

CLUSTAL W (1.74) multiple sequence alignment
ZP B1
ZP B2
ZPA S1
ZPB 3
ZPA S2
cow*
pig*
cat*

FIG. 4

Cat/pig

```
Score =  146 bits (368), Expect = 1e-34
 Identities = 80/162 (49%), Positives = 95/162 (58%), Gaps = 40/162 (24%)

Cat:   1   GCGTWVRESPGGSVLLDASYSSCYVNEWVSTTQSPGTSRPPTPASRVTPQDSHYVMIVGV 60   cat
           GCGTWV + PG S+ ++ASY  CYV EW                    DSHY+M +G+      consensus
Pig:   73  GCGTWVHKGPGSSMGVEASYRGCYVTEW----------------------DSHYLMPIGL 110  pig

Cat:   61  EGTDAAGRR-VTNTKVLRCPRNPPDQALVSSLSPSPLQNVALEAPNADLCDSVPKWDRLP 119
           E  DA G R VT TK+ +C                P+  +AL+ P   LCD+VP WDRLP
Pig:   111 EEADAGGHRTVTETKLFKC----------------PVDFLALDVPTIGLCDAVPVWDRLP 154

Cat:   120 CASSPITQGDCNKLGCCYKS-EANSCYYGNTVTSRCTQDGHF 160
           CA  PITQG+C +LGCCY S E  SCYYGNTVTSRCTQDGHF
Pig:   155 CAPPPITQGECKQLGCCYNSEEVPSCYYGNTVTSRCTQDGHF 196
```

Ferret/pig

```
Score =  162 bits (409), Expect = 1e-39
 Identities = 79/137 (57%), Positives = 90/137 (65%), Gaps = 1/137 (0%)
 Frame = +2

Ferret:26  PGSSMVLEASYSGCYVTEWVRTTQSPQMLRTPAPPSGVTPQDPHYIMLLGVEGADVTGRS 205  ferret
           PGSSM +EASY GCYVTEW                      D HY+M +G+E AD  G       consensus
Pig:   82  PGSSMGVEASYRGCYVTEW----------------------DSHYLMPIGLEEADAGGHR 119  pig

Ferret:206 TVTKTKLLKCPVDPPALDAPNADLCDSVPXWDRLPCAPSSISQRDCEKVGCCYNL-EANS 382
           TVT+TKL KCPVD  ALD P   LCD+VP WDRLPCAP  I+Q +C+++GCCYN  E  S
Pig:   120 TVTETKLFKCPVDFLALDVPTIGLCDAVPVWDRLPCAPPPITQGECKQLGCCYNSEEVPS 179

Ferret:383 CYYGNTVTSHCTQDGHF 433
           CYYGNTVTS CTQDGHF
Pig:   180 CYYGNTVTSRCTQDGHF 196
```

Ferret/cat

```
Score =  204 bits (520), Expect = 1e-52
 Identities = 97/152 (63%), Positives = 109/152 (70%), Gaps = 16/152 (10%)
 Frame = +2

Ferret:26  PGSSMVLEASYSGCYVTEWVRTTQSPQMLRTPAPPSGVTPQDPHYIMLLGVEGADVTGRS 205  ferret
           PG S++L+ASYS CYV EWV TTQSP   R P P S VTPQD HY+M++GVEG D  GR      consensus
Cat:   80  PGGSVLLDASYSSCYVNEWVSTTQSPGTSRPPTPASRVTPQDSHYVMIVGVEGTDAAGRR 139  cat

Ferret:206 TVTKTKLLKCPVDPP----------------ALDAPNADLCDSVPXWDRLPCAPSSISQR 337
            VT TK+L+CP +PP                AL+APNADLCDSVP WDRLPCA S I+Q
Cat:   140 -VTNTKVLRCPRNPPDQALVSSLSPSPLQNVALEAPNADLCDSVPKWDRLPCASSPITQG 198

Ferret:338 DCEKVGCCYNLEANSCYYGNTVTSHCTQDGHF 433
           DC K+GCCY  EANSCYYGNTVTS CTQDGHF
Cat:   199 DCNKLGCCYKSEANSCYYGNTVTSRCTQDGHF 230
```

FIG. 5a

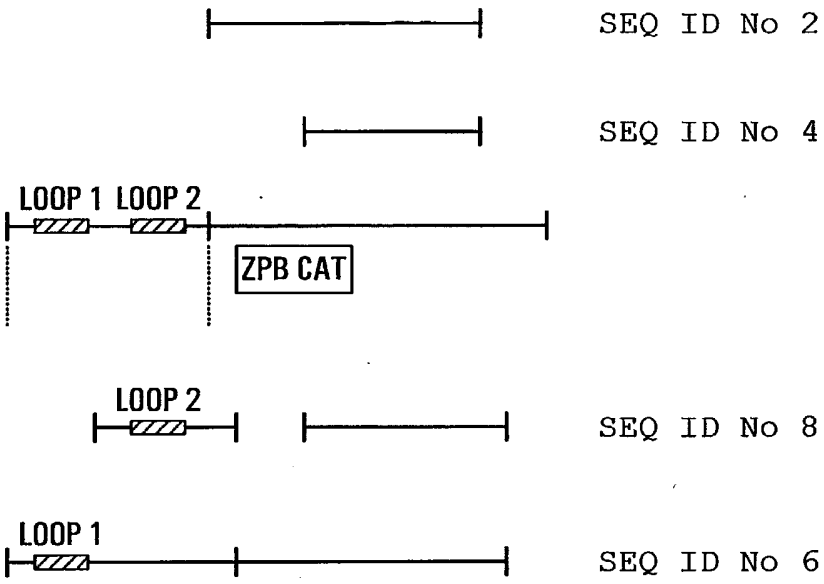
Dog/Cat

```
Score =  134 bits (337), Expect = 2e-31
 Identities = 64/85 (75%), Positives = 68/85 (79%)

Dog: 1   LRCPRNPPDPTLLSSLSYSPDQNRALDVPNADLCDFVPVWDRLPCVPSPITEEDCKKIGC 60     dog
         LRCPRNPPD L+SSLS SP QN AL+ PNADLCD VP WDRLPC  SPIT+ DC K+GC       consensus
Cat: 146 LRCPRNPPDQALVSSLSPSPLQNVALEAPNADLCDSVPKWDRLPCASSPITQGDCNKLGC 205    cat

Dog: 61  CYNLEVNFCYYGNTVTSHCTQDGHF 85
         CY  E N CYYGNTVTS CTQDGHF
Cat: 206 CYKSEANSCYYGNTVTSRCTQDGHF 230
```

FIG. 5b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SEQ ID No 4
LOOP 1
LOOP 2
ZPB CAT
LOOP 2
SEQ ID No 8
LOOP 1
SEQ ID No 6

FIG. 6

<110> Brown, Robert
Mansour, Marc
Pohajdak, William

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<130> 78961-12

<160> 2

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caccacccct tcctgaaacc caggctggac cccttactct ggaactcaag 150

attgccaaag ataagcacta tgaatcctat tacactgcca gtgactaccc 200

agtggtgaag ctgcttcggg atcccattta cgtggaggtg tctatccgcc 250

acagaacaga cccctacctg gggctgttcc tccagcactg ttgggccaca 300

cccagcctaa acccccaaca tcagcgccag tggcccatgc tggtcaatgg 350

ctgcccttа 359

<210> 2
<211> 119
<212> PRT
<213> Mustela putorius furo

<400> 2

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1 5 10 15

Ser Tyr Leu Ile Ser Ser Asn Ala Ser Gln Val Asn Val Gln Ile
20 25 30

Phe Thr Leu Pro Pro Pro Leu Pro Glu Thr Gln Ala Gly Pro Leu
35 40 45

```
Thr Leu Glu Leu Lys Ile Ala Lys Asp Lys His Tyr Glu Ser Tyr
                50                  55                  60

Tyr Thr Ala Ser Asp Tyr Pro Val Val Lys Leu Leu Arg Asp Pro
                65                  70                  75

Ile Tyr Val Glu Val Ser Ile Arg His Arg Thr Asp Pro Tyr Leu
                80                  85                  90

Gly Leu Phe Leu Gln His Cys Trp Ala Thr Pro Ser Leu Asn Pro
                95                  100                 105

Gln His Gln Arg Gln Trp Pro Met Leu Val Asn Gly Cys Pro
                110                 115
```